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Molecular nature of methicillin-resistant *Staphylococcus aureus* derived from explosive nosocomial outbreaks of the 1980s in Japan

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Abstract Community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA) with Panton-Valentine leukocidin (PVL) genes is increasing worldwide. Nosocomial outbreak-derived (hospital-acquired) MRSA (HA-MRSA) in Japan in the 1980s was also largely PVL$^+$. PVL$^+$ HA-MRSA and CA-MRSA shared the same multi-locus sequence type (ST30) and methicillin resistance cassette (SCC*mec*IV), but were divergent in oxacillin resistance, *spa* typing, PFGE analysis or *clfA* gene analysis. PVL$^+$ HA-MRSA, which probably originated in PVL$^+$ *S. aureus* ST30, was highly adhesive (carrying *cna* and *bbp* genes), highly-toxic (carrying luk_{PV} and *sea* genes) and highly drug-resistant. PVL$^+$ HA-MRSA was once replaced by other PVL$^-$ HA-MRSA (e.g., ST5), and is re-emerging as CA-MRSA.

Keywords: Community-acquired MRSA; Hospital-acquired MRSA; Nosocomial outbreak; Panton-Valentine leukocidin; Adhesin; Resistance

1. Introduction

Since at least 1960, and especially in the 1980s, methicillin-resistant *Staphylococcus aureus* (MRSA) has caused large, life-threatening nosocomial outbreaks worldwide [1]. In the late 1990s, another type of MRSA called community-acquired MRSA (CA-MRSA) also became a major concern worldwide [2,3]. CA-MRSA is found among members of a particular community who do not otherwise exhibit established risk factors for hospital-acquired MRSA (HA-MRSA) infections [1,3–5]. CA-MRSA has recently been isolated even from hospitals [6–8].

A common feature of CA-MRSA is the presence of the Panton-Valentine leukocidin (PVL) gene [3,9]. PVL has been shown to be associated with skin and soft tissue infections (SSTIs) such as severe furunculosis, cellulitis, and cutaneous abscess, and also with sepsis and osteomyelitis [9,10]. In addition, CA-MRSA is associated with community-onset pneumonia (post-influenza, necrotizing pneumonia with high mortality) in children and young adults [11–13]. PVL induces polymorphonuclear cell death by necrosis or by apoptosis [14]. Thus, CA-MRSA is considered highly virulent.

CA-MRSA also carries a type IV methicillin-resistance locus, called staphylococcal cassette chromosome *mec*IV (SCC*mec*IV), in many cases, and exhibits a low level of oxacillin resistance, compared with HA-MRSA [3,9,15]. CA-MRSA has been shown to originate from PVL$^+$ methicillin-susceptible *S. aureus* (MSSA) by acquiring SCC*mec* [16].

CA-MRSA is composed of multiple MRSA clones with distinct genetic backgrounds, as evidenced by (e.g.) multi-locus sequence typing (ST) [3]. CA-MRSA has also been shown to be continent-specific in some ST types; e.g., ST1 and ST8 mainly associated with the United States, and ST80 mainly associated with Europe [3]. In contrast, ST30 type is seen worldwide, including Oceania, the United States, Europe, Japan, and Brazil [3,15,17].

MRSA from "hospital MRSA panics" in the 1980s in Japan had not been examined for the presence of the PVL gene. In this study, we demonstrate that such nosocomial outbreak-derived HA-MRSA in Japan was largely PVL$^+$. We also show the molecular nature of PVL$^+$ HA-MRSA, in comparison with recent CA-MRSA and recent major HA-MRSA (PVL$^-$ New York/Japan ST5 clone).

2. Materials and methods

2.1. Bacterial strains

Seventeen MRSA and 11 MSSA strains were isolated in 1985 and 1986 from blood, sputum, feces, or other specimens of hospitalized patients in Tokyo. They were randomly chosen for antibiotic and antiseptic resistance examinations, and were stored at −80 °C. MRSA-infected patients included those who suffered from pneumonia and post-operative MRSA enteritis. Fifty-nine MRSA strains isolated from 1990 to 1992 were those from blood or cerebrospinal fluid of hospitalized patients in Niigata; they were stored at −80 °C. Three hundred seventy-nine MRSA strains isolated from 2000 to 2005 were those from blood, cerebrospinal fluid, sputum, pleural effusion, or urine of hospitalized patients (in NICUs, ICUs and other hospital wards) in Tokyo and Niigata. Eighteen MRSA strains were from patients with bullous impetigo or cutaneous abscess from 2003 to 2005. CA-MRSA ST30 strains used were NN1 (isolated from bullous impetigo in 2003) and NN12 (isolated from abscess in 2004) from Japan [15], USA1100 from the United States (provided by L.K. McDougal and L.L. McDonald), HT20030336 from the Netherlands and HT20010466 from Australia (provided by J. Etienne).

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Abbreviations: MRSA, methicillin-resistant *Staphylococcus aureus*; CA-MRSA, community-acquired MRSA; HA-MRSA, hospital-acquired MRSA; PVL, Panton-Valentine leukocidin; MLST, multi-locus sequence typing; SCC*mec*, staphylococcal cassette chromosome *mec*

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In this study, CA-MRSA was defined as MRSA isolated from outpatients who had no history of hospitalization within the past one year, and presented no other established risk factors for MRSA infections, such as surgery, residence in a long-term care facility, dialysis, or indwelling percutaneous medical devices and catheters [1,3–5]. HA-MRSA was defined as MRSA isolated from hospitalized patients 48 h or later after hospitalization.

S. aureus RN2677 [18], which was resistant to rifampin (100 μg/ml) and novobiocin (6 μg/ml), and restriction-negative, was used as a plasmid-free strain (recipient) in plasmid transfer experiments.

2.2. Molecular typing

Multi-locus sequence typing (MLST) was performed using seven housekeeping genes, as previously described [19]. An allelic profile (allele no.) was obtained from the MLST website (http://www.mlst.net/) and the ST data were further analyzed using eBURST-software [20] to determine a clonal complex (CC), to which each ST belonged. *spa* (staphylococcal protein A gene) typing was performed as previously described [21]. The *spa* type was determined using a public *spa* type data base (http://tools.egenomics.com/). Detection of the accessory gene regulator (*agr*) allele group was done by PCR with the reported primers, as previously described [22]. The SCC*mec* types (I–IV) were analyzed by PCR as previously described [23], using reference strains. In the case of SCC*mec*IV, three subtypes (IVa, IVb, and IVc) were further analyzed by PCR with the reported primers, as previously described [24].

2.3. Virulence gene analysis

Forty-one staphylococcal virulence genes were detected by PCR using the previously reported primers. The targeted genes were 3 leukocidin genes [25], 5 hemolysin genes [25], 16 staphylococcal enterotoxin (SE) genes [25–30], 1 putative staphylococcal enterotoxin (SEU) gene [31], 3 exfoliative toxin (ET) genes [26,32], an exotoxin-like gene cluster [33], the epidermal cell differentiation inhibitor (EDIN) gene [25] and 11 adhesin genes [34–36].

2.4. Drug resistance gene analysis

Resistance genes were detected by PCR. They included genes for methicillin resistance [23], penicillin resistance [37], cadmium resistance [38], aminoglycoside resistance [37,39,40], macrolide resistance [37] and tetracycline resistance [41–43].

2.5. Coagulase typing

The coagulase type of MRSA and MSSA strains was examined using a staphylococcal coagulase antiserum kit (Denka Seiken, Tokyo, Japan) in accordance with the manufacturer's instructions.

2.6. Susceptibility testing

Susceptibility testing of bacterial strains was done by agar dilution method with Mueller–Hinton agar according to previous procedures [44,45]. The final concentrations of antimicrobial agents were from 0.002 to 128 μg/ml.

2.7. Plasmid transfer and analysis

MRSA strains (donors) were mated with *S. aureus* RN2677 (recipient) on membrane filters, as previously described [18]. Transconjugants were selected for both donor resistance markers (cadmium acetate at 10 μg/ml, kanamycin at 10 μg/ml, erythromycin at 10 μg/ml, or tetracycline at 2 μg/ml) and recipient resistance markers (novobiocin at 5 μg/ml). Plasmid was isolated using a Qiagen Plasmid Midi kit (Qiagen, Hilden, Germany) and lysostaphin (Wako Pure Chemicals, Osaka, Japan), according to the instructions of the manufacturer, and analyzed by agarose gel electrophoresis. In some experiments, plasmid DNA was introduced into *S. aureus* RN2677 by electroporation using a Gene Pulser II electroporator (Bio-Rad, Tokyo, Japan), according to the manufacturer's instructions. Briefly, RN2677 cells were grown at 37 °C in brain heart infusion broth (BHI broth, Difco, Sparks, MD, USA) to a log phase (OD_{600} of 0.3). Bacterial cells from 100 ml culture were harvested, washed with 40 ml of cold sterilized water twice, suspended in 10 ml of cold 10% (v/v) glycerol, and finally in 0.5 ml of cold 10% (v/v) glycerol. Competent cells (50 μl) thus made were mixed with 1 μl of plasmid DNA, and the mixture was subjected to electroporation in a 0.2 cm electrode spacing cuvette (Bio-Rad) with a setting (100 Ω, 2.5 kV, 25 μF). One ml of BHI broth was then added, and then incubated for 90 min at 37 °C. Resistance plasmid-containing clones were selected on agar plates, as above.

2.8. Pulsed-field gel electrophoresis (PFGE) and computer analysis

For PFGE analysis, total bacterial DNA was extracted from MRSA or MSSA and digested with *Sma*I [46]. The digested DNA was applied on PFGE (1.2% agarose). Computer-assisted analysis of the PFGE patterns was performed using a program called Molecular Analyst Finger Printing PLUS (Bio-Rad), according to the UPGMA clustering algorithm [47].

2.9. Assay for mRNA expression of mecA

Bacteria were cultured in BHI broth to a log phase, and RNA was isolated using RiboPure™-Bacteria (Ambion, Austin, TX, USA). Then, cDNA was synthesized using a reverse transcription reactions SuperScript™ First-Strand Synthesis System for an RT-PCR kit (Invitrogen, Carlsbad, CA, USA), followed by quantitative real-time PCR assay. To amplify for the 83-bp 16S rRNA and 155-bp *mecA* gene sequences, the 16S rRNA primers [48] and the primers MECA-F1 and MECA-R1 [49] were used, respectively. A real-time PCR assay was performed using SYBR Green I dye (Applied Biosystems, Foster City, CA, USA), following the manufacturer's directions, in triplicate. The average number of *mecA* copies was divided by the average number of 16S rRNA copies to obtain each normalization value.

2.10. Analysis of mecA product PBP2′ levels

Bacteria were grown in BHI broth and the amount of PBP2′ in the 1×10^8 cells were measured using an MRSA-screen kit (Denka Seiken) in accordance with the manufacturer's instructions. Two-fold serial dilutions of the bacterial suspension were made and the highest dilution to obtain positive results in the assay was used as a titer for each bacterial sample.

2.11. Sequence analysis of virulence or drug resistance genes

PCR primers used for gene amplification to determine the nucleotide sequence included primers PVL-1 to 4 [49] for the PVL gene ($luk_{PV}SF$), 5′-ATCGTGCATTGTCGTTTG (S6F) and 5′-CCCTAATTAACAACCTACCTT (S10R) for the entire clumping factor A gene (*clfA*), 5′-CTTATATTGACCCTGAAAATG (S4F) or 5′-AAGGTGATTTAGCTTTACGTTC (S17F) and 5′-CCCTAATTAACAACCTACCTT (S10R) or 5′-TAGCCTCATTTTTATTTGAAGC (S17R) for region R of *clfA*, 5′-GCTTATCAGTTGATGATGCG (SCC*mec*F) and 5′-ATGAAAATCACCATTTTAGCTG (SCC*mec*R) for SCC*mec*IVc, 5′-GATATGACGATTCCAATGAC (*mecA*F) and 5′-TAAGGGAGAAGTAACAGCAC (*mecA*R) for *mecA*, and 5′-TCCAGTTGAAACTCTTGC (S8F) and 5′-CACCTATAATTAAACTATTCATC (S6R) for the penicillinase (PCase) gene region carrying three genes *blaI*, *R*, *Z*.

3. Results

3.1. Distribution of the PVL genes among MRSA in the past and at present

For hospital isolates, a large portion of MRSA (23.5%) in the 1980s was PVL^+ (Table 1); the four PVL^+ strains detected were designated as 80s-1, 80s-2, 80s-3 and 80s-4. In addition, 1 of 11 MSSA strains was also PVL^+; this PVL^+ strain was designated as 80s-S. In the 1990s, only 2 out of 59 MRSA (3.4%) were PVL^+; these two PVL^+ strains were designated as 90s-1 and 90s-2. No PVL^+ MRSA was detected in the 2000s (<0.3%).

For community isolates, 1 (strain NN1) of 16 MRSA strains (6.3%) isolated from patients with bullous impetigo and one (strain NN12) of two MRSA strains from patients with cutaneous abscess were PVL^+ (Table 1).

Table 1
Frequency of PVL$^+$ clones in MRSA isolated in hospitals or community

Isolation year	PVL$^+$/total	
	MRSA isolated from hospitalized patients	MRSA isolated from the community
1985–1986[a]	4/17 (23.5%)	–[b]
1990–1992	2/59 (3.4%)	–[b]
2000–2005	0/379 (<0.3%)	1/16 (6.3%)[c] 1/2[d]

[a]PVL$^+$/total in MSSA was 1/11.
[b]Data were not available.
[c]MRSA was isolated from patients with bullous impetigo.
[d]MRSA was isolated from patients with cutaneous abscess.

3.2. Characteristics of PVL$^+$ HA-MRSA and PVL$^+$ MSSA in comparison with CA-MRSA

In order to investigate evolutionary relationships and the characteristics of virulence and drug resistance, four PVL$^+$ HA-MRSA strains (strains 80s-1 to 4) and one PVL$^+$ MSSA strain (strain 80s-S) isolated in the 1980s, two PVL$^+$ HA-MRSA strains (strains 90s-1 and 2) isolated in the 1990s and CA-MRSA strains isolated in the 2000s were examined for genotypes, virulence genes and drug resistance genes or PCase plasmids (Table 2). The PVL$^+$ HA-MRSA and PVL$^+$ MSSA strains all belonged to ST30, similar to CA-MRSA. They also shared the same characteristics of *agr*3, coagulase type IV, and SCC*mec*IV (except for PVL$^+$ MSSA strain 80s-S, which lacked SCC*mec*).

There was divergence between PVL$^+$ HA-MRSA (or PVL$^+$ MSSA) and CA-MRSA. The *spa* type of PVL$^+$ HA-MRSA (and PVL$^+$ MSSA) was type43, while that of CA-MRSA was type19 or 654 (although those *spa* sequence profiles show a similarity to each other, as shown in Table 2). And, although both PVL$^+$ HA-MRSA and CA-MRSA included two subtypes of SCC*mec*IV (IVa and IVc), only the PVL$^+$ HA-MRSA group had an unknown subtype (IVx) other than IVa, IVb or IVc.

Regarding toxin genes, PVL$^+$ HA-MRSA and PVL$^+$ MSSA strains possessed *hlb* (for β-hemolysin) and *sea* (for SEA) in many cases, in contrast to CA-MRSA. PVL$^+$ HA-MRSA, PVL$^+$ MSSA and CA-MRSA strains all possessed *egc* (enterotoxin gene cluster) consisting of five genes (*seg*, *sei*, *sem*, *sen* and *seo*) plus *seu*, except for strain 80s-2 (which lacked *sen*).

Regarding adhesin genes, PVL$^+$ HA-MRSA, PVL$^+$ MSSA and CA-MRSA strains all possessed 10 of the 11 genes examined (except for CA-MRSA strains NN1 and NN12, which lacked *icaA* [for biofilm formation]); all the strains lacked *fnbB* (for fibronectin-binding protein B).

As for drug resistance, the PVL$^+$ HA-MRSA strains of the 1980s all manifested high levels of oxacillin resistance (MIC, $\geqslant$ 128 μg/ml), in contrast to CA-MRSA (MIC, 32 μg/ml) (although the oxacillin resistance levels of PVL$^+$ HA-MRSA of the 1990s were low). A penicillin- and cadmium-resistant plasmid (PCase plasmid) was present in all the PVL$^+$ HA-MRSA and CA-MRSA strains, except for PVL$^+$ HA-MRSA strain 80s-2. Such self-transmissible PCase plasmids of PVL$^+$ HA-MRSA, but not of CA-MRSA, conferred to its hosts (strain RN2677) additional multiple drug resistance (kanamycin and streptomycin resistance, and erythromycin/clindamycin resistance in some cases). Some tetracycline, gentamicin or kanamycin resistance was also separately transferred to RN2677.

Thus, a comparison of PVL$^+$ HA-MRSA and present CA-MRSA (Table 2) showed that although the two PVL$^+$ MRSA clones shared many common characteristics (such as ST30, *agr*3, SCC*mec*IV, coagulase type IV and the presence of PVL genes), there was divergence between them; only PVL$^+$ HA-MRSA exibited the *spa*43 type and SCC*mec*IVx (unknown) subtype, and had hemolysin gene *hlb*, enterotoxin (superantigen) gene *sea* and a multiple drug-resistant PCase plasmid. There were no virulence genes, which were present in CA-MRSA, but not in PVL$^+$ HA-MRSA. Analysis of PVL$^+$ MSSA (Table 2) demonstrated that it closely resembled PVL$^+$ HA-MRSA with only some differences, indicating that PVL$^+$ HA-MRSA evolved from PVL$^+$ MSSA.

3.3. Comparison with a major HA-MRSA clone in the 2000s

Next, to gain an understanding of the virulence or drug resistance characteristics of PVL$^+$ HA-MRSA of the 1980s and 1990s, comparison was also made with a major HA-MRSA clone (New York/Japan clone: ST5, SCC*mec*II, PVL$^-$) in Japan, isolated in the 2000s (Table 2).

PVL$^+$ HA-MRSA of the 1980s and 1990s and HA-MRSA of the 2000s shared similar characteristics such as the presence of toxin genes (*hlb* and *sea*) and a high oxacillin resistance level (MIC, $\geqslant$ 128 μg/ml) (Table 2). In addition, *tetM* (for minocycline [and teteracycline] resistance) was found only in these MRSA groups.

However, there was divergence; e.g., PVL$^+$ HA-MRSA lacked toxin genes *tst* (for toxic shock syndrome toxin 1) and *sec* (for SEC), and possessed enterotoxin gene *seu* in *egc* and adhesin genes *cna* (for collagen-binding protein) and *bbp* (for bone sialoprotein-binding protein), in contrast to HA-MRSA of the 2000s. Unexpectedly, no multiple drug-resistant PCase plasmid was found in HA-MRSA of the 2000s.

Thus, PVL$^+$ HA-MRSA was as virulent as present HA-MRSA in terms of the presence of *hlb* and *sea* (or *tetM*) (although they lacked *tst* and *sec*), and more adhesive in terms of the presence of *cna* and *bbp* (similar to CA-MRSA).

3.4. Cluster analysis of PFGE patterns

A computer-assisted comparison of PFGE patterns obtained with PVL$^+$ HA-MRSA, PVL$^+$ MSSA and CA-MRSA strains is shown in Fig. 1. Consistent with the *spa* typing, PVL$^+$ HA-MRSA and PVL$^+$ MSSA strains (*spa*43) constituted sub-clusters, which were distinguished from a sub-cluster of CA-MRSA (*spa*19 or 654) strains.

3.5. Divergence in the clfA gene sequences

The entire sequences or region R ([Asp-Ser]-repeating region) sequences of the *clfA* gene of PVL$^+$ HA-MRSA, PVL$^+$ MSSA and CA-MRSA were determined, and compared with the reported *clfA* sequence of *S. aureus* Newman or to each other (Fig. 2). The entire sequences of the *clfA* gene of PVL$^+$ HA-MRSA strain 80s-2 and CA-MRSA strain NN1 showed 100% similarity in the regions of S (signal peptide region), A (region including a ligand-binding domain), W (wall-anchoring, spanning region), M (membrane-spanning region) and C (cytoplasmic tail region consisting of positively charged amino acid residues) (Fig. 2A). However, in region R, there were insertions (or deletions) showing a marked divergence.

Region R sequence analysis clearly distinguished three groups: a PVL$^+$ HA-MRSA/PVL$^+$ MSSA group, a

Table 2
Characteristics of PVL$^+$ MSSA and PVL$^+$ HA-MRSA in the past and CA-MRSA and HA-MRSA at present

Type, gene, or resistance	PVL$^+$ MSSA isolated in the 1980s	PVL$^+$ HA-MRSA isolated in:						CA-MRSA isolated in the 2000s		CA-MRSA isolated from foreign countries			HA-MRSA isolated in the 2000s	
		the 1980s				the 1990s								
	80s-S	80s-1	80s-2	80s-3	80s-4	90s-1	90s-2	NN1	NN12	USA 1100 (USA)	HT2003 0336 (Netherlands)	HT2001 0466 (Australia)	E6 (from NTED)[a]	I6 (from TSS)[b]
Types														
CC[c]	30	30	30	30	30	30	30	30	30	30	30	30	5	5
ST[d]	30	30	30	30	30	30	30	30	30	30	30	30	5	5
spa	43 (WGKAK AOMQ)	43 (WGKAK AOMQ)	43 (WGKAK AOMQ)	43 (WGKAK AOMQ)	43 (WGKAK AOMQ)	43 (WGKAK AOMQ)	43 (WGKAK AOMQ)	19 (XKAK AOMQ)	19 (XKAK AOMQ)	19 (XKAK AOMQ)	654 (XKB AOMQ)	19 (XKAK AOMQ)	2 (TJMBM DMGMK)	24 (TJMEM DMGMK)
agr	3	3	3	3	3	3	3	3	3	3	3	3	2	2
SCC*mec* type	–	IVx[e]	IVc	IVa	IVa	IVx[e]	IVc	IVc	IVc	IVa	IVc	IVa	IIa	IIa
Coagulase type	IV	IV	IV	IV	IV	IV	IV	IV	IV	IV	IV	IV	II	II
Virulence genes														
Leukocidins														
luk$_{PV}$SF	+[f]	+[f]	+[f]	+[f]	+[f]	+	+	+[f]	+	+[f]	+[f]	+[f]	–	–
lukE-lukD	+	+	+	+	+	+	+	+	+	+	+	+	+	+
lukM	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Hemolysins														
hla	+	+	+	+	+	+	+	+	+	+	+	+	+	+
hlb	+	–	+	+	+	–	+	–	–	–	–	–	+	+
hlg	+	+	+	+	+	+	+	+	+	–	+	+	+	+
hlg-v	–	–	–	–	–	–	–	–	–	–	–	–	–	+
hld	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Staphylococcal enterotoxins														
tst	–	–	–	–	–	–	–	–	–	–	–	–	+	+
sea	–	+	+	–	+	+	+	–	–	–	–	–	–	+
seb	–	–	–	–	–	–	–	–	–	–	–	–	–	–
sec	–	–	–	–	–	–	–	–	–	–	–	–	+	+
sed	–	–	–	–	–	–	–	–	–	–	–	–	–	–
see	–	–	–	–	–	–	–	–	–	–	–	–	–	–
seg	+	+	+	+	+	+	+	+	+	+	+	+	+	+
seh	–	–	–	–	–	–	–	–	–	–	–	–	–	–
sei	+	+	+	+	+	+	+	+	+	+	+	+	+	+
sej	–	–	–	–	–	–	–	–	–	–	–	–	–	–
sek	–	–	–	–	–	–	–	–	–	–	–	–	–	–
sem	+	+	+	+	+	+	+	+	+	+	+	+	+	+
sen	+	+	–	+	+	+	+	+	+	+	+	+	+	+
seo	+	+	+	+	+	+	+	+	+	+	+	+	+	+

sep	−	−	−	−	−	−	−	−	−	−	−	−	−	−
seq	−	−	−	−	−	−	−	−	−	−	−	−	−	−
seu	+	+	+	+	+	+	+	+	+	+	+	+	−	−
Exfoliative toxins														
eta	−	−	−	−	−	−	−	−	−	−	−	−	−	−
etb	−	−	−	−	−	−	−	−	−	−	−	−	−	−
etd	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Other toxins														
set	+	+	+	+	+	+	+	+	+	+	+	+	+	+
edin	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Adhesions														
clfA	+	+	+	+	+	+	+	+	+	+	+	+	+	+
clfB	+	+	+	+	+	+	+	+	+	+	+	+	+	+
fnbA	+	+	+	+	+	+	+	+	+	+	+	+	+	+
fnbB	−	−	−	−	−	−	−	−	−	−	−	−	−	−
cna	+	+	+	+	+	+	+	+	+	+	+	+	−	−
eno	+	+	+	+	+	+	+	+	+	+	+	+	+	+
icaA	+	+	+	+	+	+	+	−	−	+	+	+	+	+
icaD	+	+	+	+	+	+	+	+	+	+	+	+	+	+
ebpS	+	+	+	+	+	+	+	+	+	+	+	+	+	+
fib	+	+	+	+	+	+	+	+	+	+	+	+	+	+
bbp	+	+	+	+	+	+	+	+	+	+	+	+	−	−
Drug resistance[g] and genes														
mecA (MIC of OXA, μg/ml)	− (0.25)	+ (⩾ 256)	+ (128)	+ (⩾ 256)	+ (128)	+ (32)	+ (32)	+ (32)	+ (32)	+ (32)	+ (32)	+ (32)	+ (⩾ 256)	+ (⩾ 256)
PCase plasmid	−	+ [h]	−	+ [h]	+ [h]	+ [h]	+ [h]	+ [h]	+ [h]	+ [h]	+ [h]	+ [h]	+ [h]	−
Carried genes (resistance)														
blaZ (PC)	−	+	−	+	+	+	+	+	+	+	+	+	+	−
aph(3′)-IIIa (KM)	−	+	−	+	+	+	+	−	−	−	−	−	−	−
aadE (SM)	−	+	−	+	+	+	+	−	−	−	−	−	−	−
ermB (EM, CLDM)	−	−	−	+	+	+	−	−	−	−	−	−	−	−
cadD,X (Cd)	−	− [i]	−	− [i]	− [i]	− [i]	− [i]	+	+	+	− [i]	+	+	−
Other transferred genes (resistance)														
tetK (TC)	+ [j]	−	−	+ [j]	+ [j]	−	+ [j]	−	+ [k]	−	−	−	−	−
aac(6′)-aph(2″) (GM, KM)	−	−	−	−	−	−	−	−	+ [k]	−	−	−	−	+ [l]
Other genes (resistance)[m]														
aph(3′)-IIIa (KM)	−	−	−	−	−	+	−	−	−	−	−	−	−	−
ant(4′)-I (KM)	−	−	−	+	−	−	−	−	−	−	−	−	−	+
aac(6′)-aph(2″) (GM, KM)	−	+	+	+	+	+	−	−	−	−	−	−	−	−
aadE (SM)	−	−	−	−	−	−	−	−	−	−	−	−	−	−
ermA (EM, CLDM)	+	+(i) [n]	+(i) [n]	+	+	−	(+)[o]	−	−	−	−	−	+	+

(*continued on next page*)

Table 2 (*continued*)

Type, gene, or resistance	PVL$^+$ MSSA isolated in the 1980s	PVL$^+$ HA-MRSA isolated in:						CA-MRSA isolated in the 2000s		CA-MRSA isolated from foreign countries			HA-MRSA isolated in the 2000s	
		the 1980s				the 1990s								
	80s-S	80s-1	80s-2	80s-3	80s-4	90s-1	90s-2	NN1	NN12	USA 1100 (USA)	HT2003 0336 (Netherlands)	HT2001 0466 (Australia)	E6 (from NTED)[a]	I6 (from TSS)[b]
ermB (EM, CLDM)	+	–	–	–	–	–	–	–	–	–	–	–	–	–
tetM (TC, MINO)	–	+	–	–	–	–	–	–	–	–	–	–	+	+

[a]NTED, neonatal toxic shock syndrome-like exanthematous disease. NTED is caused by MRSA infection in neonates in a neonatal intensive care unit (NICU). It frequently occurs at 2–4 days old and the major symptoms are fever and subsequent exanthema. The fever persists for about one day and decreases spontaneously. The exanthema is systemic erythema (2–3 mm erythemas later fuse) spreading over the face, trunk, all four limbs, palms, and soles, spontaneously diminishing within 2–3 days. A superantigen TSST-1 (encoded by *tst*) is the major cause of NTED [50]. Strain E6 was isolated from the umbilical blennorrhoea of an infant (female aged 5 days old) with NTED in Niigata [51].

[b]TSS, toxic shock syndrome. TSS is a life-threatening staphylococcal infection usually for adults [52]. Although most of HA-MRSA in hospitals in Japan possess *tst*, the development of TSS is rare in hospitals (the reason for this is not known). However, when TSS develops, it is very severe, and may lead to death. TSST-1 is the major causative toxin of TSS. Strain I6 was isolated from a patient (male aged 59 years old) with TSS in Niigata [15].

[c]CC, clonal complex.

[d]ST, multi-locus sequence type.

[e]Type IV with unknown subtypes (other than IVa, IVb or IVc).

[f]PVL gene sequences were identical to the NN1 sequence (AB186917); GenBank Accession Nos. for strains 80s-S, 80s-1 to 4, USA1100, HT20030336 and HT20010466 were AB245448, AB245449, AB245450, AB245451, AB245452, AB245453, AB245454 and AB245455, respectively.

[g]Resistance: OXA, oxacillin; PC, penicillin G (or ampicillin); KM, kanamycin; SM, streptomycin; EM, erythromycin; CLDM, clindamycin; TC, tetracycline; GM, gentamicin; MINO, minocycline; Cd, cadmium.

[h]PCase (penicillin- and Cd- resistant) plasmid (size in kb) for strains 80s-1, 80s-3, 80s-4, 90s-1, 90s-2, NN1, NN12, USA1100, HT20030336, HT20010466 and strain E6 were pAC80s1 (37 kb), pAC80s3 (42 kb), pAC80s4 (44 kb), pAC90s1 (46 kb), pAC90s2 (29 kb), pAC00s1 (32 kb), pAC00s2 (32 kb), pAC00s3 (29 kb), pAC00s4 (29 kb), pAC00s5 (29 kb) and pAC00s6 (25 kb), respectively. Of those, pAC80s1, pAC80s3, pAC80s4, pAC00s1, pAC00s2, pAC00s3 and pAC00s5 were identified by filter mating (conjugal transfer) to *S. aureus* RN2677 (transfer frequency was 2.0×10^{-9} or lower), and pAC90s1, pAC90s2, pAC00s4, and pAC00s6 were transferred to RN2677 by electroporation (of plasmid DNA).

[i]Cd-resistance gene was negative for *cadD,X*.

[j]TC resistance was transferred to RN2677 by filter mating (for strains 80s-S, 80s-3 and 80s-4) or by electroporation (for strain 90s-2). TC resistance plasmids were detected for strains 80s-S, 80s-3, 80s-4 and 90s-2 (size 4.6, 4.1, 4.1 and 4.6 kb, respectively).

[k]Strain NN12 carried the transmissible pGKT1 plasmid (size 41 kb) carrying *tetK* and *aac(6′)-aph(2″)*.

[l]Strain E6 carried the *aac(6′)-aph(2″)* gene, which could be transferred to RN2677 by electropolation.

[m]Drug resistance was not transferred to RN2677 by conjugation or electroporation.

[n]Inducible clindamycin resistance.

[o]Clindamycin intermediate resistance.

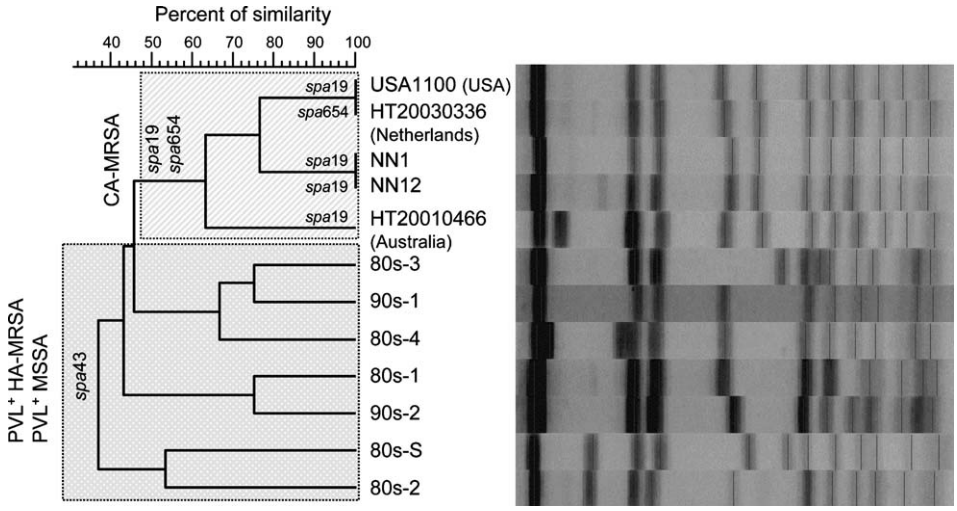

Fig. 1. PFGE analysis of PVL$^+$ HA-MRSA, PVL$^+$ MSSA and CA-MRSA, and dendrogram constructed by computer-assisted comparison. PFGE patterns (right side) were obtained after DNA digestion with *Sma*I. Strain numbers are shown at the right side of the dendrogram (or at the left side of the PFGE patterns). In the dendrogram, sub-clusters consisting of *spa*43 strains and those consisting of *spa*19 and 654 strains are separately marked with shading.

CA-MRSA group and a foreign CA-MRSA group (Fig. 2A). The CA-MRSA group had segment S-3, the PVL$^+$ HA-MRSA/PVL$^+$ MSSA group had segments S-1 and S-2 and the foreign CA-MRSA group had segments S-1 and S-3.

In addition, strain 90s-2 showed a strain-specific deletion of segment S-4 (Fig. 2A, *1). Strains 80s-1 and 90s-1 exhibited a non-synonymous substitution G → T (Asp → Tyr) (Fig. 2A, *2), and strains USA1100 and HT20010466 exhibited a synonymous substitution A → C (Fig. 2A, *3).

The sequences of the divergent segments (S-1 to 4) are shown in Fig. 2B. S-1 was a four-times repetition of the $(Asp\text{-}Ser)_3$ unit (18 bp), with substitution (Asp → Gly, Asn or Thr) at six positions (Fig. 2B). S-2 to S-4 were a twice repetition of the $(Asp\text{-}Ser)_3$ unit (18 bp), with one substitution (Asp → Glu) in S-4.

3.6. Divergence in the SCCmecIV sequences

Since PVL$^+$ HA-MRSA of the 1980s showed a higher level of oxacillin resistance than did present CA-MRSA (Table 2), the entire sequences of SCC*mec*IVc of PVL$^+$ HA-MRSA strain 80s-2 (MIC of oxacillin, 128 μg/ml) and CA-MRSA strain NN1 (MIC of oxacillin, 32 μg/ml) were determined, and compared with each other (Fig. 3). The SCC*mec*IVc sequence at the 3′ end (including *mecA* and *ccrB* genes) was well conserved with only four nucleotides changed, while the sequence at the 5′ end (including the *ccrA* gene) was slightly divergent.

Consistent with high oxacillin MIC levels, PVL$^+$ HA-MRSA strains (80s-1 to 4) manifested higher *mecA* transcription and PBP2′ production, compared with CA-MRSA strains (NN1 and NN12) (Table 3). PVL$^+$ HA-MRSA strains possessed Shine-Dalgarno (SD) sequence with G → T substitution (with a lower ΔG value) that may affect translation efficiency (Fig. 3 and Table 3). There was animo acid substitution in the *mecA* product (PBP2′) at position 146 or 246 (Table 3), but not contributing to the different level of oxacillin resistance (Asn and Gly at positions 146 and 246 were seen in both PVL$^+$ HA-MRSA and CA-MRSA).

Since the regulatory genes *blaI* (for repressor) and *blaR* (for a sensor-transducer) on a PCase plasmid, which regulate the expression of *blaZ* (for PCase), may also affect expression of the *mecA* gene on SCC*mec*, the gene sequences of *blaI* and *blaR* (and *blaZ* for PCase) on PCase plasmids of strains 80s-3 and NN1 were determined. They were identical to each other (or showed only substitution between homologous amino acids) (Table 3), indicating no regulatory effects on the oxacillin resistance of these *blaI* and *blaR* genes.

4. Discussion

In this study, the authors demonstrated that MRSA from hospitalized patients in the 1980s (when "hospital MRSA panics" occurred in Japan) was largely PVL$^+$. To our knowledge, this study provides the first molecular characterization of such PVL$^+$ HA-MRSA. Since CA-MRSA, described in the United States and Europe, is considered to be a highly virulent MRSA [3,11,12], and the PVL gene of PVL$^+$ HA-MRSA was identical to those of CA-MRSA, PVL$^+$ HA-MRSA could also have been virulent.

Regarding diseases, for instance, decubitus, pneumonia, bacteremia and post-operative MRSA enteritis in elder (or adult) patients [54–56] and bacteremia and abscess in pediatric patients [57] were frequently found in the late 1980s and the early 1990s, but have drastically decreased recently. Of PVL$^+$ HA-MRSA examined, one strain (80s-4) from the 1980s was associated with pneumonia (no records were available for three remaining strains), and two strains from the 1990s were derived from blood. PVL$^+$ HA-MRSA might not be associated with MRSA enteritis, because two HA-MRSA strains isolated in the 1980s from MRSA enteritis (and examined in this study) were PVL$^-$; the strains were positive for at least *tst*, *sea* and *sec*.

PVL$^+$ HA-MRSA belonged to ST30, just like CA-MRSA, but the two MRSAs were unambiguously divergent, as evidenced by *spa* typing, PFGE analysis, *mecA* SD sequence analysis, sequence analysis of the *clfA* R region, or oxacillin MIC analysis. The former has been replaced by the latter. PVL$^+$ HA-MRSA seems to have originated in PVL$^+$ MSSA ST30 in the 1980s or before. A possible ancestor (PVL$^+$ MSSA

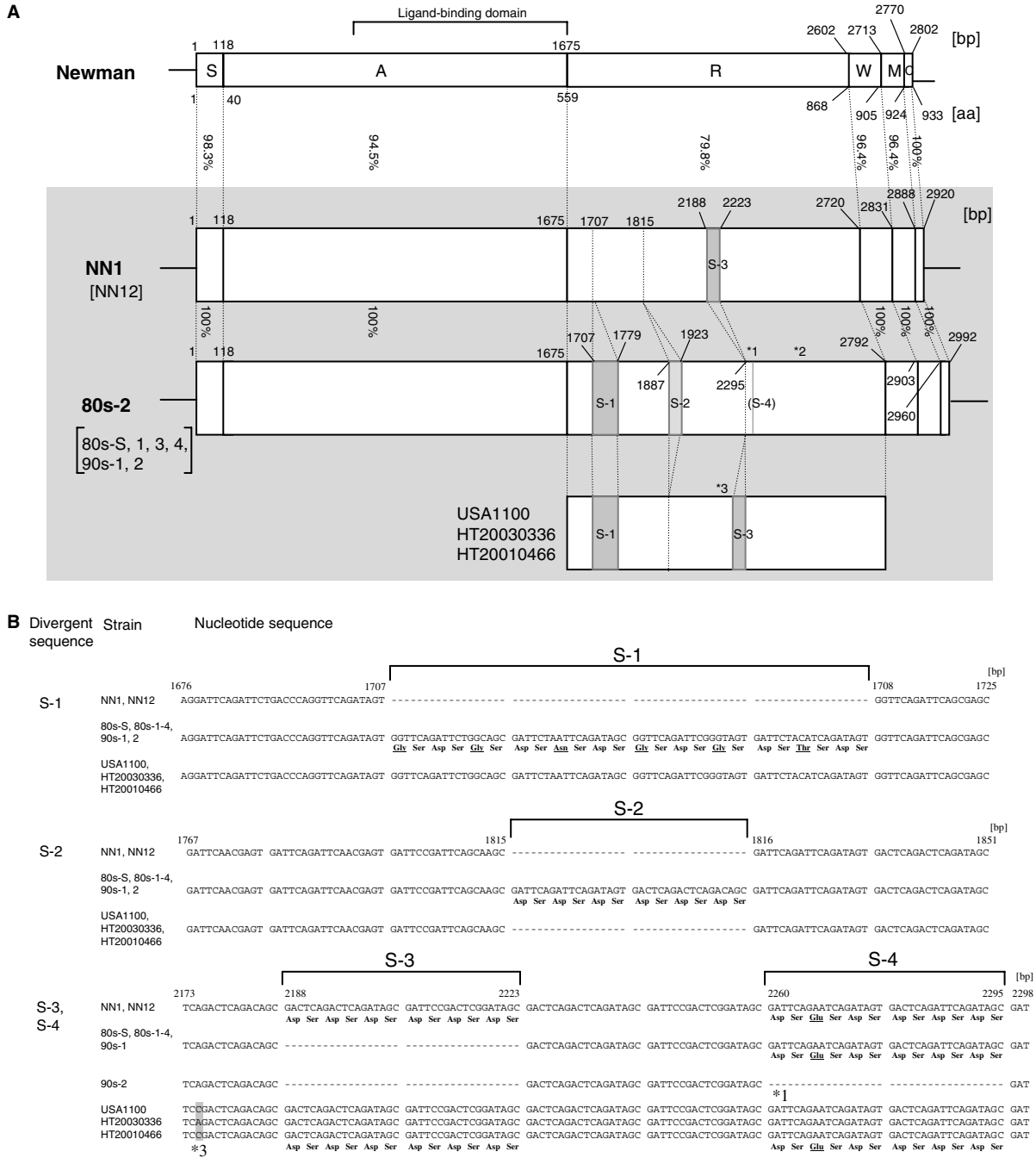

Fig. 2. Sequences of the *clfA* gene of PVL$^+$ HA-MRSA, PVL$^+$ MSSA and CA-MRSA. The GenBank Accession Nos. for the entire *clfA* gene sequence of strains NN1 and 80s-2 are AB245456 and AB245457, respectively. In case of strains NN12, 80s-S, 80s-1, 80s-3, 80s-4, 90s-1, 90s-2, USA1100, HT20030336 and HT20010466, only region R sequences were determined (GenBank Accession Nos. AB245467, AB245458, AB245459, AB245460, AB245461, AB245462, AB245463, AB245464, AB245465 and AB245466, respectively) and drawn in the figure. In A, the locations of the regions S, A, R, W, M and C in the *clfA* gene are drawn based on the Newman strain data [53]. *1, deletion of 36 bp (corresponding to twice repetition of the 6 amino acids unit) in strain 90s-2; *2, non-synonymous substitution (GAC → TAC, Asp → Tyr; at nucleotide position 2590 of corresponding 80s-2 sequence) in strains 80s-1 and 90s-1; *3, synonymous substitution (TCA → TCC) in strains USA1100 and HT20010466. In B, nucleotide sequences of the divergent segments (S-1 to 4) are presented. The corresponding amino acid residues are drawn below the nucleotide sequence (amino acids that differed from Asp-Ser are underlined).

ST30) of recent CA-MRSA has not been identified yet; ST types of recent PVL$^+$ MSSA in Japan (so far examined) are ST50 and ST508.

In hospitals in Japan, it seems that PVL$^+$ HA-MRSA ST30 was a dominant clone in the early 1980s. Then, this clone was replaced by an other PVL$^-$ HA-MRSA clone (New York/Japan ST5) in the 1990s. The present dominant HA-MRSA clone is still New York/Japan ST5. This replacement seems to have occurred in many hospitals in Japan, since our previous data (and reports [58]) showed that coagulase type IV MRSA was dominant in the early 1980s and then replaced by coagulase type II MRSA. Consistent with this, in this study,

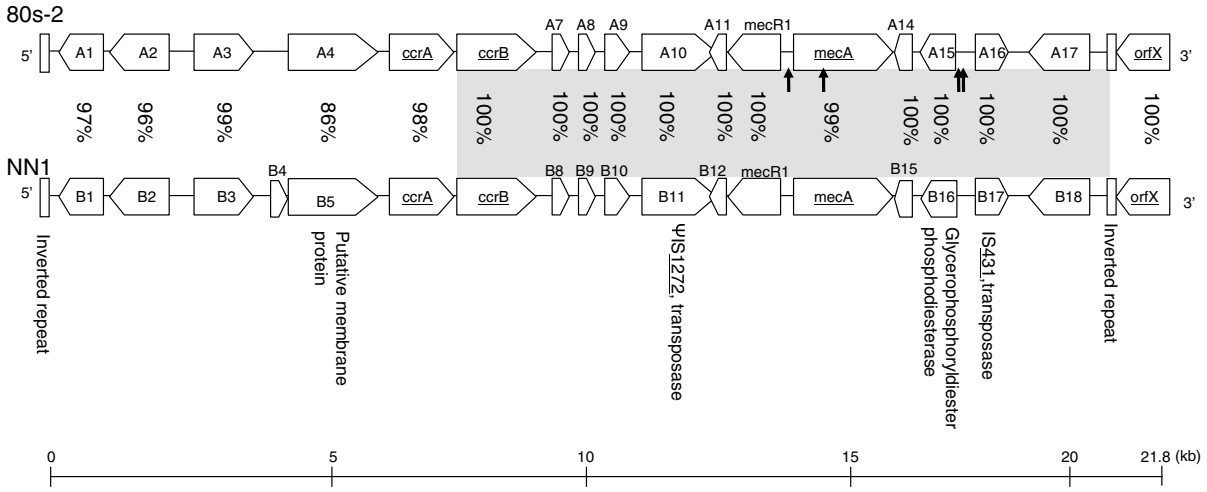

Fig. 3. SCC*mec*IVc sequences of PVL$^+$ HA-MRSA (strain 80s-2) and CA-MRSA (strain NN1). The GenBank Accession Nos. for the entire SCC*mec*IVc sequence of strains NN1 and 80s-2 are AB245470 and AB245471, respectively. Arrows indicate a single base substitution. Conserved regions in the SCC*mec*IVc are marked with shading.

Table 3
Oxacillin-resistance-related characteristics of PVL$^+$ HA-MRSA and CA-MRSA

Strain	MIC of oxacillin (μg/ml)	Relative *mecA* transcript levels (*mecA*/16s rRNA)	PBP2′ production (titer)	SD sequence for *mecA*	Sequence of PBP2′ [a]		PCase plasmid genes		
					146	246	*blaI*	*blaR*	*blaZ*
MSSA									
80s-S	0.25	0.0	<1	–	–	–	–	–	–
PVL$^+$ HA-MRSA									
80s-1	⩾ 256	5.2 ± 1.2	4	AAGGAGG (−16.0 kcal)	Lys (AAA)	Glu (GAA)	+	+	+
80s-2	128	10.2 ± 3.8	4	AAGGAGG (−16.0 kcal)	Asn (AAT)	Glu (GAA)	–	–	–
80s-3	⩾ 256	13.1 ± 1.2	4	AAGGAGG (−16.0 kcal)	Asn (AAT)	Gly (GGA)	+[b]	+[b,c]	+[b]
80s-4	128	4.4 ± 0.7	8	AAGGAGG (−16.0 kcal)	Asn (AAT)	Gly (GGA)	+	+	+
CA-MRSA									
NN1	32	0.8 ± 0.6	2	AAGGAGT (−12.0 kcal)	Asn (AAT)	Gly (GGA)	+[b]	+[b,c]	+[b]
NN12	32	0.1 ± 0.02	2	ND[d]	ND[d]	ND[d]	+	+	+

[a]GenBank Accession Nos. for the *mecA* gene sequence of strains 80s-1 to 4 and NN1 are AB221119, AB221120, AB221121, AB221122 and AB221124, respectively.
[b]GenBank Accession Nos. for the *blaI*, *blaR* and *blaZ* gene sequences of strains 80s-3 and NN1 are AB245469 and AB245468, respectively.
[c]There was a non-synonymous substitution at nucleotide position 271 (GCA, Ala in strain 80s-3; ACA, Thr in strain NN1).
[d]ND, not determined.

the coagulase type of PVL$^+$ HA-MRSA ST30 was type IV, and that of New York/Japan MRSA ST5 was type II.

Also recently, the PVL$^+$ HA-MRSA ST30 which left the hospitals may be re-emerging as a CA-MRSA ST30 in the community, probably losing some virulence genes such as *hlb*, *sea* and *icaA* and drug resistance genes on a PCase plasmid to become CA-MRSA. These genes found with PVL$^+$ HA-MRSA may be associated with hospital infection.

The reason for this dynamic MRSA replacement in Japan is not known. However, there is a possibility that PVL$^+$ HA-MRSA infection caused a marked increase of antibodies against (e.g.) PVL and bacterial surface adhesins (such as those for collagen-binding or bone sialoprotein-binding; genes *cna* and *bbp*) in individuals, which in turn suppressed PVL$^+$ HA-MRSA infection and allowed emergence of PVL$^-$, *cna*$^-$ and *bbp*$^-$ New York/Japan clone in hospitals, and that CA-MRSA (PVL$^+$, *cna*$^+$ and *bbp*$^+$), evolved from PVL$^+$ HA-MRSA, is now appearing in the community (especially in children and adolescents) where PVL$^+$, *cna*$^+$ and *bbp*$^+$ MRSA infection was rare. Further study is necessary to examine this hypothesis.

In this study, we also investigated *clfA* gene sequences to look at divergence between PVL$^+$ HA-MRSA and CA-MRSA. The R domain of clumping factor A (ClfA) consists of Asp-Ser repeats, and appears to serve as a "stalk" allowing presentation of the ligand-binding domain (A) at the bacterial surface for ligand interactions [53]. In region R, thrice repetition of Ser-Asp (18 bp long) consisted of a further repeating unit [53]. Insertions (or deletions) observed in this study were mainly twice or four times repetition of such an 18-bp unit, indicating a possibility that the 36-bp long unit ([Asp-Ser]$_6$ unit) plays a role in insertion (or deletion) recognition, and that (Asp-Ser)$_6$ is a basic unit in the functional "stalk" structure.

Interestingly, this analysis also distinguished CA-MRSA ST30 in Japan from CA-MRSA ST30 from other countries. Thus, sequencing of region R of *clfA* could be useful for bacterial typing.

PVL$^+$ *S. aureus* adheres to collagens and laminin [13]. In this study, we demonstrated that PVL$^+$ HA-MRSA and CA-MRSA carried both *cna* and *bbp*, while recent HA-MRSA (New York/Japan clone) did not. Collagen-binding protein (CBP) plays a role in adherence to exposed type I and IV collagens after epithelial damage due to viral infection (e.g., influenza) [11–13]. Further, bone sialoprotein-binding protein (BSBP) plays a role in adherence to the cells or hydroxyapatite of bone tissue [59,60]. BSBP$^+$ staphylococci are associated with hematogenously spread osteomyelitis and arthritis, especially in young persons [59]. Indeed, CA-MRSA strain NN12 was isolated from an 18-year-old female high school student (a basketball player) with an abscess and bacteremia and subsequently with osteomyelitis. We concluded that the CA-MRSA ST30 clone (and therefore PVL$^+$ HA-MRSA) is highly virulent in terms of tissue adherence.

In addition, PVL$^+$ HA-MRSA (but not CA-MRSA) possessed *hlb* and *sea*. *sea* and *lukE-lukD* are associated with post-antibiotic diarrhea [61], and *sea* is associated with severity of infection (sepsis and shock) [62]. Thus, PVL$^+$ HA-MRSA is a genotypically highly-adhesive (CBP$^+$, BSBP$^+$) and highly-toxic/superantigenic (PVL$^+$, SEA$^+$) clone.

PVL$^+$ HA-MRSA in the 1980s exhibited a high level of oxacillin resistance and carried a multiple drug-resistant PCase plasmid. Minocycline resistance (*tetM*) was also found in this group. These results also suggest a strong association of PVL$^+$ HA-MRSA with nosocomial infection. The reason why PVL$^+$ HA-MRSA in the 1980s (or recent HA-MRSA) exhibited a high level of oxacillin resistance is not known. However, there is a possibility that the divergent 5′ side region of SCC*mec* regulates the *mecA* transcription.

In conclusion, HA-MRSA in the 1980s (when "hospital MRSA panics" occurred in Japan) was largely PVL$^+$. PVL$^+$ HA-MRSA and CA-MRSA shared the same clonal characteristics such as ST30 or SCC*mec*IV, but the two were apparently divergent in *spa* typing, PFGE analysis, *clfA* region R sequence analysis, oxacillin resistance levels, PCase plasmid analysis and others. PVL$^+$ HA-MRSA was highly adhesive (possessing CBP and BSBP), highly toxic/superantigenic (being positive for PVL, SEA and β-hemolysin) and highly drug-resistant (exhibiting a high level of oxacillin-resistance and possessing a multiple drug-resistant PCase plasmid). This genotype is unique to PVL$^+$ HA-MRSA, and not found in recent CA-MRSA or HA-MRSA. PVL$^+$ HA-MRSA was replaced by the PVL$^-$ New York/Japan HA-MRSA ST5 clone in the 1990s, and now is re-emerging as CA-MRSA in the community losing some virulence genes.

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